United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine

Update on PPQ Diagnostics for SOD:
Diagnostics Procedures Approved by APHIS and Diagnostic Analyses Performed by APHIS PPQ for *Phytophthora ramorum*
Revised 10/21/04

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At present, diagnostics for *Phytophthora ramorum* are a pyramidal design to accommodate the large number and variability of samples generated as a result of trace forwards and the national survey. A brief summary of how these tests function can be found at www.aphis.usda.gov/ppq/ispm/sod/diagnostics.html.

1. **ELISA**

The approved ELISA is test produced by Agdia, Inc. (see, www.aphis.usda.gov/ppq/ispm/sod/ELISAprotocol.html). This immunologically-based assay is designed to detect any species of *Phytophthora* and is also reported to detect some species of *Pythium.* The intent of using ELISA as a 'pre-screen' is to reduce the number of samples that will need to be processed for subsequent tests. In general, ELISA tests are relatively high throughput. Very recently, Agdia released a modified extraction buffer that reduces high background levels seen with certain hosts, especially *Syringa*. Samples that are tested by ELISA and found to be above a background threshold are to be tested further, as described below (see also, Table 1). Samples that are below the threshold level do not need to be tested further. The current diagnostic protocol does not require the use of ELISA. However, it is important to keep in mind that this is a national program and that there are limited resources for diagnoses. The ELISA test is intended as triage, thereby significantly reducing the number of samples that need to be tested further, and thus reducing pressure on later stages of the diagnostic process. All laboratories are strongly encouraged to use the Phytophthora ELISA for screening of all samples.

2. **Culturing of *Phytophthora ramorum***

Current methods to be used to attempt to isolate the organism are described at www.aphis.usda.gov/ppq/ispm/sod/cultureprotocol.html. Laboratories that follow this procedure must perform a preliminary assessment of the results of the attempted isolation. If they determine that an organism is present that appears to be *P. ramorum*, based on morphology (a presumptive positive), then that culture (on V8 agar) is sent to Dr. Mary Palm (PPQ National Mycologist, USDA APHIS PPQ NIS, Room 329, Building 011A, BARC-West, 10300 Baltimore Blvd., Beltsville, MD 20705-2350, 301-504-5327) for confirmation. Up to 10 leaf sections can be placed on each plate, but they must be from the same sample. If other *Phytophthora* species or other organisms or if nothing grows on the plate(s), the result is considered to be negative for *P. ramorum*. i.e., unless *P. ramorum* is isolated, the result of the culture assay is "negative".

The current diagnostic protocol does not require culturing of *P. ramorum* by diagnostic labs from field samples or ELISA positive samples (Table 1). It is an optional test because a 'negative' *P.*

ramorum culture assay means that *P. ramorum* was not detected, but it cannot be interpreted to indicate that it is not present. This is due to the inefficiency of culturing *P. ramorum* because of host effects, environmental conditions, etc. Ideally, however, all samples should be tested by culturing and PCR, because it is desirable to have a diagnosis based on two or more independent tests. We recognize that this is not always possible, but performing the culture assay offers our diagnosticians certain advantages. When *P. ramorum* is successfully isolated, and the identity of that isolate confirmed, this result is diagnostic.

3. PCR for Diagnosis of *P. ramorum*

The current diagnostic protocol (as of 4/22/04) is that all samples that are ELISA positive and/or culture negative must be assayed using the validated nested PCR method by USDA or at authorized USDA laboratories. In other words, ELISA positive samples are extracted for DNA (see, www.aphis.usda.gov/ppq/ispm/sod/pcrprotocol.html), and these DNA samples **(minimum 50 µL volume)** are forwarded to the NPGBL (which at present is the only authorized laboratory) as described in the protocol (see Table 1). If the diagnostic laboratory has not performed the ELISA test (but note that screening using ELISA is highly recommended), then culture negative samples need to have DNA extracted and these extracts sent to NPGBL for analysis (Dr. Laurene Levy, USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Biotechnology Laboratory (NPGBL), Building 580-East, Powdermill Road, Beltsville, MD 20705, 301.504.7100).

The NPGBL testing protocol is as follows: After samples are received and logged into our database, the DNA is subjected to PCR tests. The Oregon State Univ. multiplex PCR test is performed, (as described in Forest Pathology, 31:275, 2001). The purpose of this test is not to determine if *P. ramorum* is present (although if the DNA of the organism is present in sufficient quantity it will detect it), but rather to determine if the DNA sample contains 'amplifiable' DNA. This is because occasionally the DNA extraction may fail or the DNA extract contains contaminants that inhibit the PCR test. In this way, we greatly reduce or eliminate false negative results of the much more sensitive validated nested PCR assay by ensuring that DNA of an acceptable level of quality is present in the sample. The other assay, assuming that amplifiable DNA is present in the sample, is the validated nested PCR test, described in www.aphis.usda.gov/ppq/ispm/sod/pcrprotocol.html. In the event that the results of this test are inconclusive, the test is repeated. If the second nested PCR test is inconclusive, the laboratory that submitted the sample is asked to obtain another, if possible. Note that procedures are in place to minimize false positive results by using an appropriate dilution of DNA.

Because of the extensive level of testing that is performed on samples, throughout the diagnostic system, steps have been taken to attempt to ameliorate potential bottlenecks. One major bottleneck is preparation of DNA from samples, and this is why this step in the process has been distributed across the network of diagnostic laboratories. A similar situation exists for culturing of the organism. Note that DNA extraction from bark or wood is challenging, and methods to do this are still under development and have not been validated to date. Therefore, there is some uncertainty associated with results obtained from these samples, and steps should be taken to confirm initial results. Additionally, the current validated PCR test is not particularly well suited to high-throughput, which is why we desire to reduce the number of samples subjected to this test by the use of ELISA prescreening. The necessity of extracting DNA from these samples requires that diagnostic laboratories to take all necessary steps to prevent contamination of the samples. Since the validated protocol can pick up extremely minute traces of *P. ramorum* DNA, sample processes that might be adequate to prevent cross-contamination for ELISA or culturing may not be sufficient to prevent contamination of the

sample for PCR. Initial handling of samples for diagnosis that may later be tested by PCR should be done to prevent possible cross-contamination.

The APHIS-PPQ program on *P. ramorum* is arguably one of the most complex ever deployed by the agency. Definitive diagnosis of *P. ramorum* is not simple or trivial, but we endeavor to deploy the best, most scientifically sound protocol available. New tests and new methods are constantly in development, and those tests most appropriate will be validated and deployed, as we are able.

Table 1. Summary of SOD diagnostic tests, and actions associated with each procedure.

Culture		Nested PCR	Action
+	Or	+	Yes
-	And	-	No
Not required	n/a	-	No
Not required	n/a	+	Yes
-	And	+	Yes
+	n/a	(optional)	Yes